MOLECULAR AND CELLULAR BIOLOGY, Dec. 1994, p. 8471–8482
0270-7306/94/$04.00+0

Vol. 14, No. 12

Selective Degradation of Early-Response-Gene mRNAs: Functional Analyses of Sequence Features of the AU-Rich Elements

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Received 24 June 1994/Returned for modification 2 August 1994/Accepted 28 August 1994

The metabolic lifetime of mRNA can be specified by specific *cis*-acting elements within mRNA. One type of element is an adenylate- and uridylate-rich element (ARE) found in the 3′ untranslated region of many highly unstable mRNAs for mammalian early-response genes (ERGs). Among the better-characterized members of the ERG family are certain genes encoding nuclear transcription factors. Of particular significance was the finding that their mRNAs decay rapidly with kinetics similar to those of c-*fos* mRNA. Our previous studies of the c-*fos* ARE-directed mRNA decay have revealed the existence in this ARE of two structurally distinct and functionally interdependent domains, termed domain I and domain II. We proposed that the c-*fos* ARE-directed decay is a two-step mechanism in which rapid shortening of the poly(A) tail leads to the decay of the mRNA body and further hypothesized that this is a general mechanism by which the ERG AREs mediate rapid mRNA degradation. To test this hypothesis and to further address the generality of the critical structural characteristics within the c-*fos* ARE, the RNA-destabilizing functions of more than 10 different AU-rich sequences from various nuclear transcription factor mRNAs have been tested. Consistent with the above-mentioned hypothesis is the observation that mRNAs carrying the functional AREs display a biphasic decay, which is characteristic of the proposed two-step mechanism. Our results indicated that the presence of AUUUA pentanucleotides in an AU-rich region does not always guarantee an RNA-destabilizing function for this region. Our results also led to the identification of a novel class of AU-rich destabilizing elements which contains no AUUUA pentanucleotide. The results of sequence comparison and functional tests revealed that a continuous U-rich sequence is a unique feature among the functional AREs. Finally, our experiments further showed that the c-*fos* ARE domain II has an RNA decay-enhancing ability upon its fusion to heterologous AU-rich regions and defined for the first time an RNA decay-enhancing element, which we termed the RDE element.

Modulation of mRNA stability provides a powerful means for controlling gene expression. The stability of mRNA can be specified by specific *cis*-acting elements within mRNA (for reviews, see references 5, 14, and 33). One type of element is an adenylate- and uridylate-rich element (ARE), found in the 3′ untranslated region of many highly unstable mRNAs for mammalian early-response genes (ERGs) whose transcription is rapidly and transiently activated by a variety of extracellular stimuli, including various growth factors (for reviews, see references 5 and 19). Of particular significance was the finding that mRNAs of these genes decay rapidly with kinetics similar to those of c-*fos* mRNA (2, 19, 24). The ARE is a loosely defined sequence element with two general features: (i) the presence of various copies of an AUUUA pentanucleotide, and (ii) a high content of uridylate and sometimes also adenylate residues. As different AREs vary considerably in their sizes and sequence contexts, it has not been clear what the precise structural and sequence features of a functional ARE are. One of the important questions that remain unanswered is whether we can assume that the presence of AUUUA motifs in an AU-rich region is sufficient evidence of a functional RNA-destabilizing element. Nonetheless, the fact that the presence of AUUUA motifs in the mRNA 3′ untranslated region has been cited as evidence of an RNA-destabilizing element has pervaded the literature. Therefore, to address the mode of action of the ARE and the basis of its specificity, it appears crucial to obtain a clear and in-depth understanding of the key sequence and structural features of the AREs that identify them as mRNA decay determinants.

We have chosen two approaches to address the relationship between structure and function for AREs. Our initial approach was to focus on the characterization of the c-*fos* ARE (12). Previously, combining extensive mutagenesis of the c-*fos* ARE with in vivo analysis of mRNA stability, we were able to identify mutations that exhibited kinetic phenotypes consistent with the biphasic decay characteristic of a two-step mechanism: accelerated poly(A) shortening leads to subsequent decay of the transcribed portion of the mRNA (12). These mutations, which affected either an individual step or both steps, all changed the mRNA stability. Our experiments further revealed the existence of two structurally distinct and functionally interdependent domains that constitute the c-*fos* ARE (Fig. 1). Domain I, which is located within the 5′ 49-nucleotide (nt) segment of the ARE and contains the three AUUUA motifs, can function as an RNA destabilizer by itself. It forms an essential core unit necessary for the c-*fos* ARE destabilizing function. Domain II is a 20-nt U-rich sequence which is located within the 3′ part of the c-*fos* ARE. Although it cannot act alone as an RNA destabilizer, this domain plays two critical roles: (i) its presence enhances the destabilizing ability of domain I by accelerating the deadenylation step, and (ii) it has a novel capacity of buffering against decay-impeding effects exerted by mutations introduced within domain I. Domain II

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FIG. 1. RNA sequence of the AU-rich regions from the 3′ UTRs of various nuclear transcription factor mRNAs. The 69-nt human c-*fos* ARE sequence is shown on the top with AUUUA motifs underlined and domain I and domain II bracketed. The AUUUA and GUUUG pentanucleotides in all the other ARSs are also underlined.

enhances decay of those messages carrying mutant domains I by accelerating rates for both steps of the mutant domain I-directed mRNA decay.

In an attempt to further our understanding of the mechanisms of ARE-directed mRNA degradation and to better address the generality of the c-*fos* ARE-directed decay, we have used a second approach in this study to carry out functional tests on more than 10 different AU-rich sequences (ARSs) from the 3′ untranslated regions (UTRs) of six other ERG transcripts, all encoding nuclear transcription factors. These include c-*myc*, *nur*77, c-*jun*, *krox*20, *jun*B, and *zif*268 (2, 19, 24). Our experiments revealed several novel findings. First, our results showed that the presence of AUUUA motifs, even in a highly AU-rich region (>65%), does not necessarily constitute evidence of a functional RNA-destabilizing element. Second, we identified an ARS which does not carry any AUUUA pentanucleotides but can still function as a potent RNA-destabilizing element. Finally, we demonstrated that the

c-*fos* ARE domain II has an RNA decay-enhancing ability by fusing it immediately downstream of heterologous AU-rich regions.

MATERIALS AND METHODS

Cell culture and DNA transfection. Culturing, transient transfection, and serum stimulation of mouse NIH 3T3 cells were performed as described previously (39).

Plasmid constructions. Various AU-rich regions (Fig. 1) were amplified by standard PCR techniques (20) with a plasmid carrying c-*jun* (3), c-*myc* (4), *nur*77 (18), *zif*268 (25), *jun*B (32), or *krox*20 (11). Sequences of the amplified fragments are shown in Fig. 1. All the PCR-amplified fragments are flanked by a *Bam*HI site at their 5′ ends and a *Bgl*II site at their 3′ ends. After *Bam*HI and *Bgl*II digestion, the fragments were introduced into the unique *Bgl*II site of plasmid pBBB, which is immediately downstream of the translation termination codon (39). To test the effect of the human c-*fos* ARE domain II on the destabilizing function of these AU-rich regions, a 33-bp *Bam*HI-*Bgl*II fragment containing this domain was prepared as described previously (12) and then subcloned into the unique *Bgl*II site of these plasmids as described above. To construct the plasmid pSVα1-GAPDH, a PCR-amplified *Bst*EII fragment spanning nucleotides 46 to 663 of the rat glyceraldehyde 3′-phosphate dehydrogenase cDNA (15) was inserted in frame into the unique *Bst*EII site of the alpha-globin coding region sequence. A poly(dT-dA) fragment encoding an 85-nt poly(A) or poly(U) sequence was prepared by *Hin*dIII and *Eco*RI digestions of the plasmid pSP64 (polyT) (9), blunt ended with Klenow enzyme, and inserted into the unique *Bgl*II site (blunt ended with Klenow enzyme) in the β-globin 3′ UTR in both orientations to create the poly(U)$_{85}$ and poly(A)$_{85}$ insertions, respectively.

Analysis of mRNA decay and deadenylation. Total cytoplasmic RNA was isolated at various times after serum stimulation of transiently transfected NIH 3T3 cells and was analyzed by an RNase protection assay or Northern (RNA) blotting as described previously (38, 39). Decay of mRNA was quantitated directly by scanning gels on a Betascope 603 blot analyzer (Betagen). When an RNase protection assay was employed, human α1-globin mRNA was detected with a 272-nt RNA probe derived from the *Eco*RI-linearized plasmid pT7α181 (39). β-Globin (BBB) mRNAs carrying various AU-rich regions were detected with a 238-nt RNA probe derived from the *Bam*HI-linearized plasmid pT7BBF (38). Transcription reactions were performed according to instructions from Promega. Labeled RNA transcripts were produced by inclusion of [α-^{32}P]UTP (800 Ci/mmol; Amersham or New England Nuclear).

Northern blotting was performed to analyze mRNA decay and mRNA deadenylation as described previously (38). To analyze the mRNA decay, a new internal control, α/GAPDH, was used in transient transfection experiments. The α/GAPDH mRNA has a size of ~1,200 nt and can be easily distinguished from the β-globin mRNA containing the AREs by Northern blotting. Electrophoresis was done on 1.4% formaldehyde agarose gels. A 123-nt single-stranded DNA ladder (BRL) was included to provide a molecular size standard. Gene-specific DNA probes were prepared by the method of random oligonucleotide priming. Labeled probes were produced by inclusion of [α-^{32}P]dCTP (>6,000 Ci/mmol; Amersham). RNase H treatment of cytoplasmic mRNA was carried out as described previously (38). Decay of mRNA was quantitated directly by scanning gels or blots on a Betascope 603 blot analyzer (Betagen).

RESULTS

The presence of AUUUA motifs in an AU-rich region does not always guarantee a functional ARE. In an attempt to establish the general principles that may identify an AU-rich region as an mRNA-destabilizing element, we sought to identify, characterize, and compare the mechanistically critical features of potent AREs. Initially, we searched for ARSs from the 3′ UTRs of six different ERG mRNAs, all of which contain at least one AUUUA motif and have a minimal AU content of >65% and a length of 60 to 150 nt. These included mRNAs from the proto-oncogene families of c-*myc*, c-*jun*, and *jun*B as well as from genes encoding certain nuclear transcription factors, such as *zif*268, *nur*77, and *krox*20. Eight ARSs that fulfill these criteria were identified (Fig. 1). It is important to emphasize that all six messages encode nuclear transcription factors and decay rapidly with kinetics similar to those of c-*fos* mRNA upon their arrival in the cytoplasm of serum-induced fibroblasts (2, 19, 24).

To test the candidate ARSs for their ability to function as RNA-destabilizing elements, they were introduced individually into a unique *Bgl*II site in the 3′ UTR of the stable β-globin mRNA (BBB mRNA) (39), whose transcription is driven by the serum-inducible c-*fos* promoter. The decay rates and the poly(A) shortening status of β-globin mRNAs carrying the various ARSs were then determined by Northern blot analysis. The use of the c-*fos* promoter to drive transcription allowed transient synthesis of mRNA to be induced by treatment of transfected NIH 3T3 cells with serum (17, 40). Time course experiments could then be performed to monitor the decay and highly synchronous poly(A) shortening of the mRNA of interest without using transcription inhibitors. As described previously (12), we used four parameters in assessing how each individual ARS behaves. (i) The overall half-life ($t_{1/2}$) measures mRNA decay starting at 70 min postinduction, when transcription from the c-*fos* promoter returned to preinduction levels. This parameter is a summation of the time required for accelerated deadenylation and the time required for the subsequent decay of the mRNA body. It served as an indicator for the general stability of an mRNA. (ii) The $t_{1/2}$ for the second step measures the decay of the mRNA body following the first phase of accelerated poly(A) shortening. The first time point chosen for calculating the $t_{1/2}$ for this step represents the time point following which the amount of mRNA starts to decrease significantly. Unlike the first parameter, $t_{1/2}$ for the second step displays first-order kinetics. (iii) The deadenylation rate measures the rate for poly(A) shortening between the 30-min time point, when mRNA with the full-length poly(A) tail has just appeared in the cytoplasm, and the time point when significant decay of the mRNA body starts to occur. (iv) The length of the poly(A) tail still retained by mRNA when significant decay of the mRNA body commences is also determined.

Initially, we focused on two ARSs which have sequence features similar to those of the c-*fos* ARE, i.e., they contain at least one copy of the AUUUA motif and a long stretch of uridylates plus some scattered short uridylate stretches. These include one from the 3′ UTR of c-*myc* mRNA and one from the *jun*B 3′ UTR (Fig. 1, c-*myc* and *jun*B#1). The c-*myc* ARS has two copies of the AUUUA motif and a stretch of 10 U residues as well as a few copies of short U stretches. The *jun*B#1 ARS has one copy of the AUUUA motif and a stretch of 10 U residues. The results show that both the c-*myc* and the *jun*B#1 ARSs have a drastic destabilizing effect, reducing the half-life of β-globin mRNA from more than 8 h to 39 and 72 min, respectively (Fig. 2). Interestingly, both ARSs appear to direct a biphasic decay characteristic of the two-step mecha-

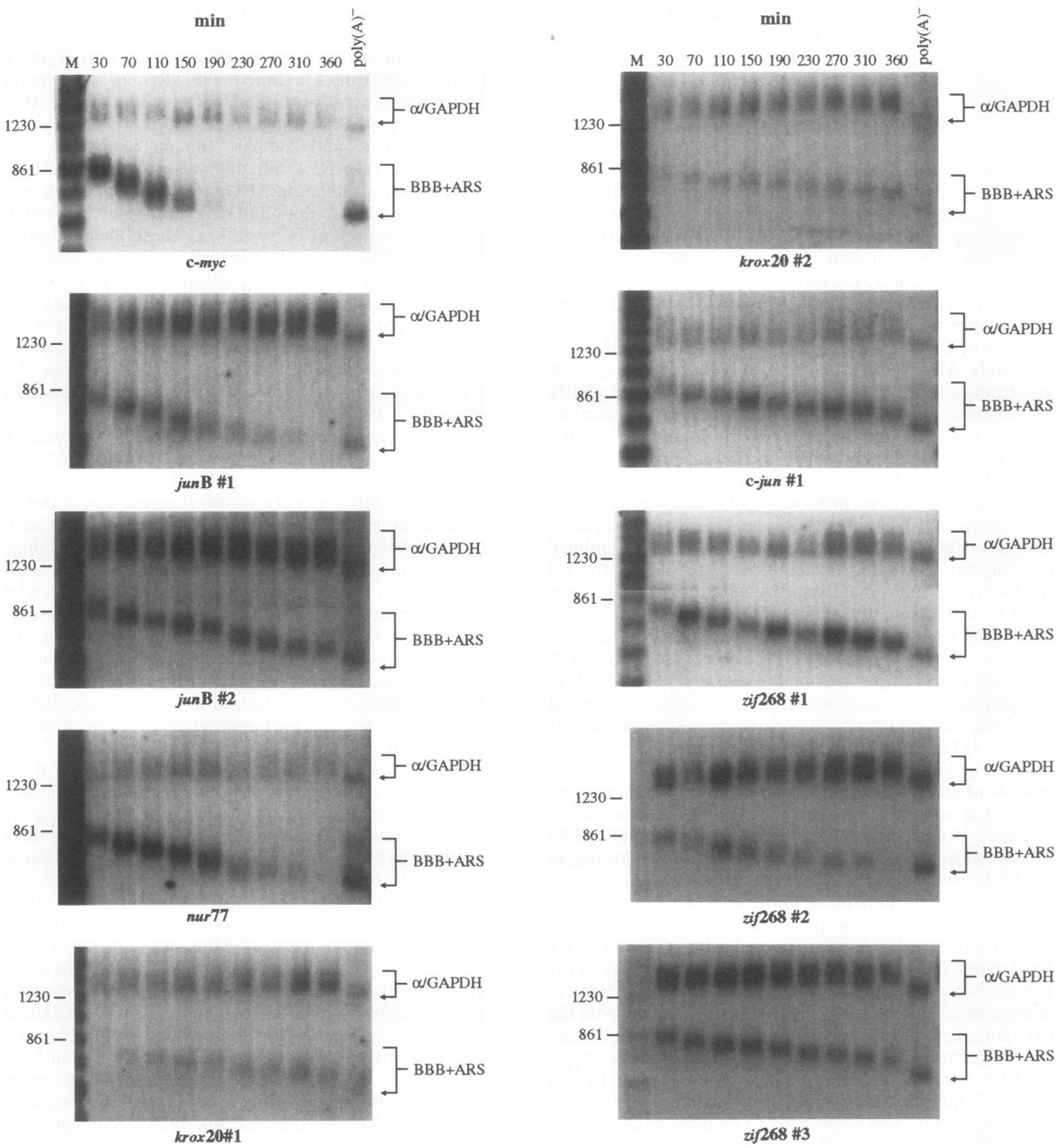

FIG. 2. Decay and deadenylation of β-globin (BBB) mRNAs carrying various ARSs. NIH 3T3 cells were transiently cotransfected with the control plasmid pSVα1-GAPDH and one of the test plasmids. Total cytoplasmic mRNA was isolated at various time intervals after serum stimulation and analyzed by Northern blotting. The times at the top are times after serum stimulation. Thirty minutes after serum induction, BBB+ARS mRNA still retained a full-length poly(A) tail. Poly(A)$^-$ RNA was prepared in vitro by treating RNA samples from 30-min time points with oligo(dT) and RNase H. M, 123-bp DNA ladder from BRL used as a molecular size standard. Note that the somewhat broad bands corresponding to the α/GAPDH mRNA in each blot are due to the poly(A) tail heterogeneity caused by the constitutive transcription of the α/GAPDH gene and subsequent unsynchronized poly(A) shortening of α/GAPDH mRNAs. Numbers on the left of each gel are nucleotides.

nism, as observed with the c-*fos* ARE (Fig. 2). Thus, these two ARSs represent two potent AREs. These results suggest that a long U stretch coupled with at least one AUUUA motif may be a major determinant of the destabilizing efficacy of the ARE.

To gain further insight into the above-mentioned possibility, six more ARSs from *jun*B, *nur*77, *krox*20, c-*jun*, and *zif*268 transcripts were tested. These include the *jun*B#2, *nur*77, *krox*20#1, *krox*20#2, c-*jun*#1, and *zif*268#1 ARSs (Fig. 1). One common characteristic of these ARSs is that they all contain one to three copies of AUUUA pentanucleotides in a

TABLE 1. Summary of half-lives and deadenylation rates for various BBB and ARS mRNAs

ARS[a]	$t_{1/2}$ (min)		Deadenylation rate (nt/min)	Poly(A) size (nt) (time point)[b]
	Overall	Second step		
c-*fos* ARE I	60 ± 26	20 ± 5	0.88	35 (190)
c-*myc*	39 ± 7	30 ± 1	1.6	60 (110)
*jun*B#1	72 ± 12	52 ± 7	1.2	59 (150)
*jun*B#2	183 ± 41	64 ± 0	0.53	36 (310)
*nur*77	135 ± 19	86 ± 3	0.75	59 (190)
*krox*20#1	>480	—[c]	0.44	34 (360)
*krox*20#2	>480	—	0.35	65 (360)
c-*jun*#1	>480	—	0.35	53 (360)
*zif*268#1	>480	—	0.4	37 (360)
*zif*268#2	179 ± 44	84 ± 5	0.69	44 (230)
*zif*268#3	411 ± 73	—	0.33	67 (360)
c-*fos* ARE I · E	35 ± 8	21 ± 1	1.2	60 (110)
c-*myc* · E	34 ± 6	29 ± 5	2.0	10 (110)
*jun*B#1 · E	51 ± 9	40 ± 4	1.5	48 (110)
*jun*B#2 · E	124 ± 27	65 ± 6	0.84	15 (230)
*nur*77 · E	51 ± 13	31 ± 4	1.1	58 (150)
*krox*20#1 · E	81 ± 20	32 ± 3	0.7	30 (270)
*krox*20#2 · E	391 ± 173	88 ± 24	0.5	50 (270)
c-*jun*#1 · E	>480	127 ± 28	0.63	54 (270)
*zif*268#1 · E	>480	128 ± 10	0.65	16 (270)
*zif*268#2 · E	83 ± 7	69 ± 3	1.2	38 (150)
*zif*268#3 · E	205 ± 74	52 ± 5	0.53	51 (270)
BBB (β-globin mRNA without ARS)	>480	—	0.40	65 (360)

[a] · E represents the c-*fos* ARE domain II which was fused immediately downstream of the ARS. c-*fos* ARE I, c-*fos* ARE without domain II.

[b] Size of the poly(A) tail at time point (in minutes) at which decay of the mRNA body occurred. 360 min, the last time point of the time course experiment.

[c] —, not detectable during the time course experiment.

context with a few scattered short (2- to 4-residue) or medium (5- to 7-residue) U stretches but lack a longer stretch of continuous U residues and a long U-rich sequence. As shown in Fig. 2 and Table 1, insertion of the *krox*20#1, *krox*20#2, c-*jun*#1, or *zif*268#1 ARS into the stable β-globin mRNA has no destabilizing effect. The β-globin transcripts carrying these ARSs all decay with a half-life greater than 8 h, and their poly(A) shortening proceeds with a rate similar to that of the β-globin mRNA (~0.4 nt/min) (Table 1). The *jun*B#2 and *nur*77 ARSs are the only two with a moderate destabilizing effect and show a somewhat higher poly(A) shortening rate (Fig. 2 and Table 1). Again, the β-globin mRNA carrying either one of these two ARSs also displayed a biphasic decay. Together, these results substantiated the above-mentioned possibility that a U stretch coupled with an AUUUA motif(s) may be a major factor in determining the destabilizing ability of the ARE but further indicated that the presence of AUUUA motifs in a context of scattered short or medium U stretches is not sufficient evidence of a functional RNA-destabilizing element. More importantly, these results demonstrated that the mere presence of AUUUA motifs in an AU-rich region does not always suffice to ensure a functional ARE.

c-*fos* domain II has an RNA decay- enhancing function when fused to heterologous AU-rich regions. Since only two (c-*myc* and *jun*B#1) of the eight ARSs we tested showed a significant destabilizing effect comparable with that of the c-*fos* ARE, we sought to identify the possible sequence differences or similarities among these ARSs that may account for the observed differences in their destabilizing abilities. As pointed out in the previous section, one prominent difference between the functioning AREs and nonfunctioning ARSs is the presence in the c-*fos*, c-*myc*, and *jun*B#1 AREs of a long stretch of U residues or a U-rich sequence. In the case of the c-*fos* ARE, a 20-nt extraordinarily U-rich domain in the 3′ portion of the ARE, termed domain II, has been shown to have a unique function (12) (Fig. 1). Its presence in the c-*fos* ARE can buffer against significant decay-impeding effects caused by various types of mutation in domain I of the c-*fos* ARE. We therefore fused the c-*fos* ARE domain II immediately downstream of the 8 ARSs we had tested to see how the manipulation might affect the destabilizing function of these ARSs.

The results of these eight fusions are summarized in Fig. 3 and 4. Interestingly, the c-*fos* ARE domain II had an enhancing effect on the RNA-destabilizing function of these heterologous ARSs. All the new hybrids showed an increased destabilizing capability, ranging from 1.4-fold to more than sixfold, when they were inserted into the 3′ UTR of β-globin mRNA. The most dramatic effect was seen in the case of *krox*20#1 (Fig. 3 and Table 1). The fusion significantly enhanced the ability of the *krox*20#1 ARS to direct both the poly(A) shortening step (0.70 nt/min) and the subsequent step for decay of the RNA body (second-step $t_{1/2}$ = 32 ± 3 min). The data showed that the c-*fos* ARE domain II not only accelerates poly(A) shortening but also increases the extent of poly(A) tail removal. As shown in Fig. 5, it reduced the length of the remaining poly(A) tail of the last detectable message in the time course experiments from a range of 30 to 65 nt to a range of 0 to 40 nt. Moreover, in nearly all cases (except for c-*myc* and *jun*B#2) it caused a significant increase of the rate for the second step (Table 1). Although the fusion of the c-*fos* ARE domain II to the c-*myc* ARE did not change the second step for the c-*myc* ARE-mediated decay, this manipulation further enhanced the deadenylation step (Table 1). The c-*fos* ARE domain II made the c-*myc* ARE and one other potent ARE, the *jun*B#1 ARE, function even better. Therefore, its ability to enhance the activity of the destabilizing element is not limited to those AU-rich regions which have no or just modest destabilizing effects. These results also indicate that mechanistically the c-*fos* ARE domain II is able to participate in either one or both steps of the ARE-directed mRNA decay. Moreover, they strongly support the possibility that AUUUA motifs coupled with a nearby long U-rich sequence or U stretch may be a major feature of those potent AREs.

The presence of the AUUUA motif is not an absolute requirement for an AU-rich region to function as an RNA-destabilizing element: identification of a non-AUUUA ARE. As demonstrated above, the presence of AUUUA motifs in the context of an AU-rich environment is not always sufficient to ensure a functional ARE. We then asked whether the presence of AUUUA motifs is always necessary for an ARS to be able to function as an RNA-destabilizing element. One way to answer this question will be to search for ARSs that contain no AUUUA motifs but are still able to function as RNA-destabilizing elements. Therefore, three non-AUUUA ARSs from the 3′ UTRs of the c-*jun* and *zif*268 messages (Fig. 1, c-*jun*#2, *zif*268#2, and *zif*268#3), which have an AU content of at least 68%, were identified for further tests. Remarkably, insertion of the c-*jun*#2 ARS into the β-globin mRNA caused a dramatic reduction of its stability, reducing its half-life from more than 8 h to just 35 min (Fig. 6). The *zif*268#2 ARS had only a moderate effect, and the *zif*268#3 ARS had no destabilizing

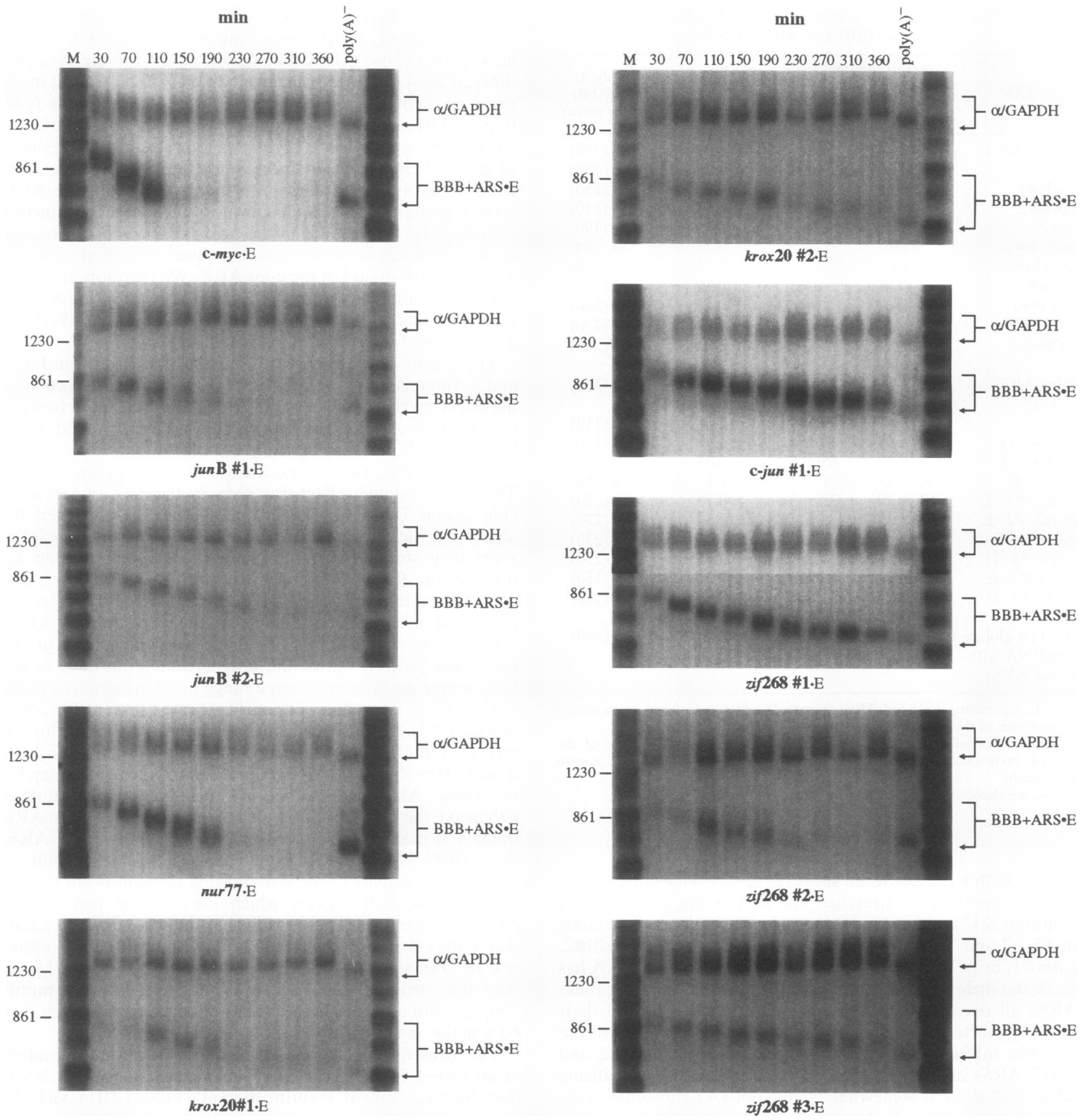

FIG. 3. Effect of the c-*fos* ARE domain II on decay and deadenylation of β-globin (BBB) mRNAs carrying various ARSs. Transient transfection, RNA isolation, and time course experiments were carried out as described in the legend to Fig. 2. The times at the top are times after serum stimulation. Poly(A)$^-$ RNA was prepared in vitro by treating RNA samples from 30-min time points with oligo(dT) and RNase H. Note that the somewhat broad bands corresponding to the α/GAPDH mRNA in each blot are due to the poly(A) tail heterogeneity caused by the constitutive transcription of the α/GAPDH gene and subsequent unsynchronized poly(A) shortening of α/GAPDH mRNAs. M, 123-bp DNA ladder from BRL used as a molecular size standard. ·E, the c-*fos* ARE domain II fused immediately downstream of the ARS. Numbers on the left of each gel are nucleotides.

ability (Fig. 2). Therefore, even though the c-*jun*#2 ARE contains no AUUUA motifs, its destabilizing ability is just as potent as that of the c-*fos* ARE. Moreover, the β-globin mRNA carrying the c-*jun*#2 ARE displayed a pattern of biphasic decay similar to that of the c-*fos* ARE. It underwent deadenylation with a rate (~2.0 nt/min) much higher than that of the stable β-globin mRNA (0.4 nt/min). The amount of mRNA started to decrease significantly after the 110-min time point. The half-life for the second step was ~25 min (Fig. 6). These results demonstrated that the presence of AUUUA

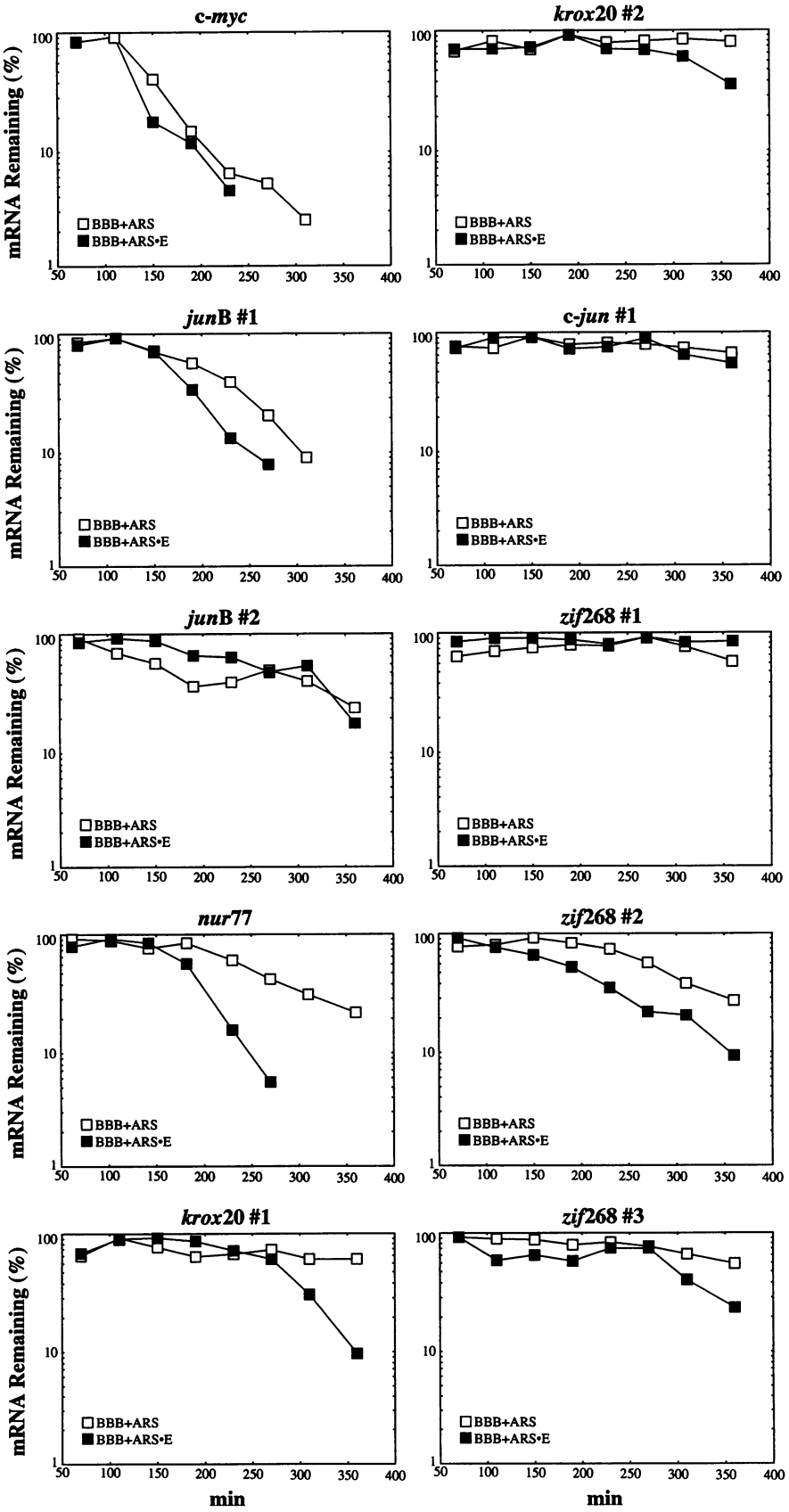

FIG. 4. The c-*fos* ARE domain II has a general decay-enhancing effect. The time course of the decay of β-globin (BBB) mRNAs carrying various ARSs as shown in Fig. 2 and 3 is plotted. Quantitation of data was obtained by scanning the radioactive blots with a Betascope blot analyzer (Betagen). The identities of the ARSs tested are indicated at the top of each graph. · E, the c-*fos* ARE domain II fused immediately downstream of the ARS.

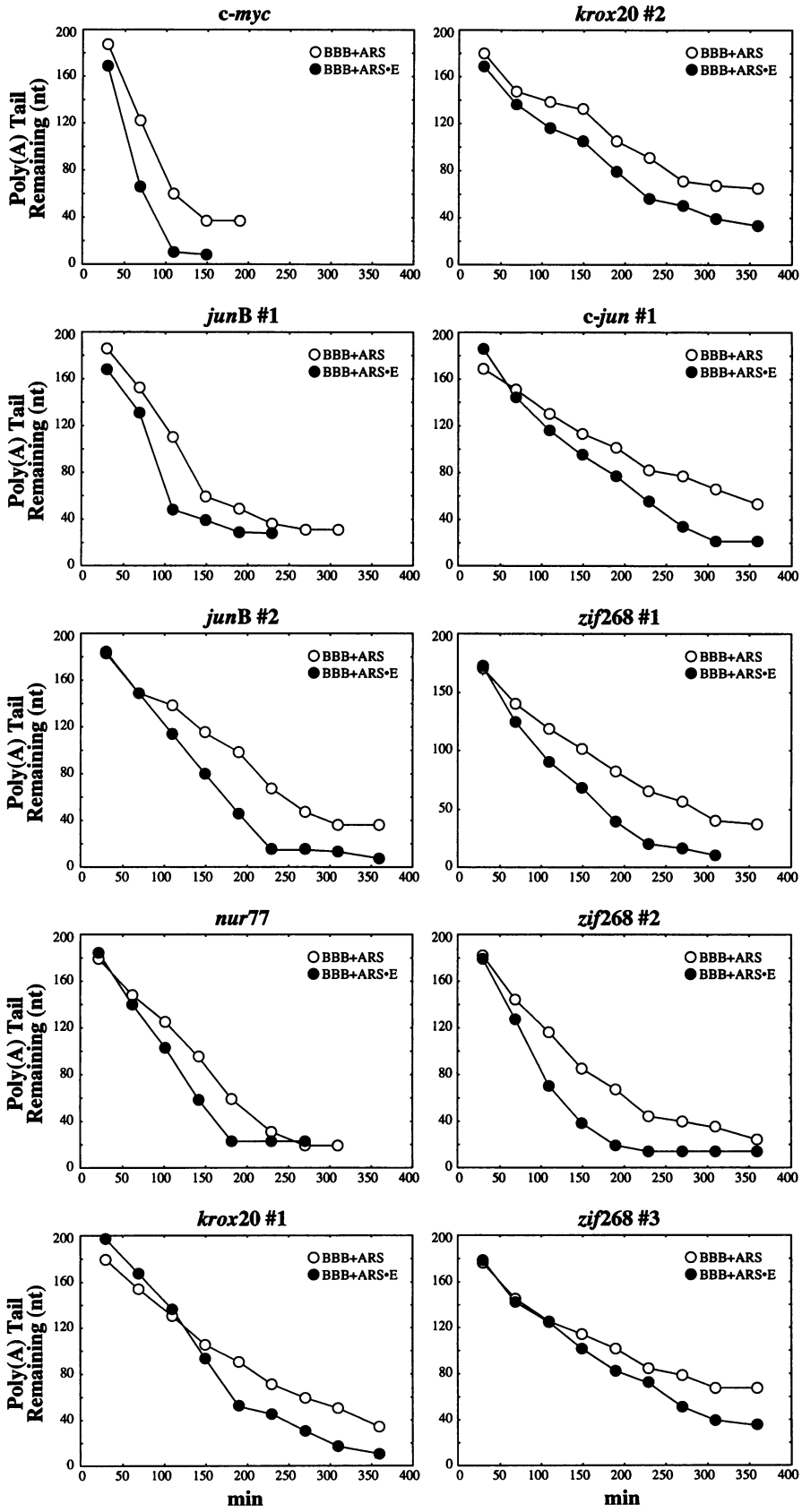

FIG. 5. The c-*fos* ARE domain II has an enhancing effect on the rate and extent of deadenylation directed by various ARSs. The time course of deadenylation of β-globin (BBB) mRNAs carrying various ARSs as shown in Fig. 2 and 3 is plotted. Quantitation of data was obtained by scanning the radioactive blots with a Betascope blot analyzer (Betagen). The identities of the ARSs tested are indicated at the top of each graph. · E, the c-*fos* ARE domain II fused immediately downstream of the ARS.

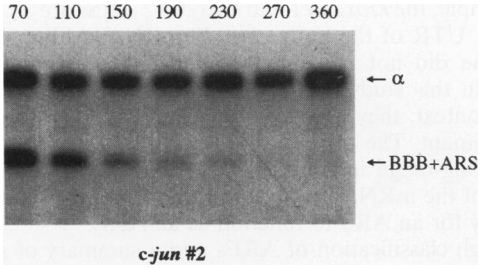

FIG. 6. Decay of β-globin mRNA carrying the c-*jun*#2 ARS. NIH 3T3 cells were transiently cotransfected with the control plasmid pSVα1 (α) (39) and the plasmid pBBB+c-*jun*#2 ARS (BBB+ARS). Total cytoplasmic mRNA was isolated at various time intervals after serum stimulation and analyzed by RNase protection and gel electrophoresis on denaturing 6% polyacrylamide gel. The times at the top are times after serum stimulation.

motifs is not an absolute requirement for all the ARSs to function as RNA-destabilizing elements.

To further explore the decay-enhancing ability of the c-*fos* ARE domain II, we fused it immediately downstream of two of the three non-AUUUA containing AU-rich regions (i.e., *zif*268#2 and *zif*268#3). As shown in Table 1, this manipulation increased the destabilizing activities of the two non-AUUUA ARSs by approximately twofold by accelerating rates for both steps of the decay (Fig. 4 and 5). Taken together, these results demonstrated that the c-*fos* ARE domain II has an enhancing effect on the RNA destabilizing functions of not only AUUUA-containing ARSs but also non-AUUUA ARSs.

Analysis of degradation of β-globin mRNA carrying either the poly(U) or poly(A) insertion. To an extreme approximation, the AU-rich region can be represented by either a poly(A) or a poly(U) sequence. Therefore, a DNA fragment containing 85 bp of poly(dT-dA) was inserted into the unique *Bgl*II site in the β-globin 3′ UTR in both orientations, and the resulting constructs were analyzed by transient transfection.

TABLE 2. Summary of half-lives and deadenylation rates for β-globin mRNA carrying the poly(U) or poly(A) insertion[a]

mRNA	$t_{1/2}$ (min)		Deadenylation rate (nt/min)	Poly(A) size (nt) (time point)[b]
	Overall	Second step		
BBB+poly(U)	175 ± 27	116 ± 0	0.53	28 (190)
BBB+poly(A)	168 ± 36	113 ± 26	0.83	9 (190)

[a] Data were obtained by scanning the radioactive gels and blots, in Fig. 7 with a Betascope blot analyzer (Betagen).

[b] Size of the poly(A) tail that remained at time point (in minutes) at which decay of the mRNA body occurred.

The results of RNase protection analyses show that both homopolymers have a modest destabilizing effect, reducing the half-life of stable β-globin mRNA from more than 8 to approximately 3 h (Table 2). Further analyses of the poly(A) shortening status of the β-globin mRNAs carrying the two insertions by Northern blotting revealed very interesting results. The β-globin mRNA with either a poly(A) or a poly(U) insertion also displayed a biphasic decay. The BBB+poly(U) mRNA underwent deadenylation with a rate (0.53 nt/min) slightly higher than that of the stable β-globin mRNA (0.4 nt/min), while the BBB+poly(A) mRNA underwent poly(A) shortening with a rate (0.83 nt/min) significantly higher than that of the β-globin mRNA. In both cases, no significant decay of the mRNA body was observed until the poly(A) tail was shortened to ~28 nt for BBB+poly(U) or to ~9 nt for BBB+poly(A) at the 190-min time point. The mRNA body then decayed with first-order kinetics. The half-lives for this second step are about 2 h for both BBB+poly(U) and BBB+poly(A). Interestingly, both homopolymer inserts are the only two among the various ARSs we tested that have the ability to direct the complete removal of the poly(A) tail in the 6-h time course experiment. In the case of BBB+poly(A) mRNA, to avoid the cleavage of the internal 85-nt poly(A)

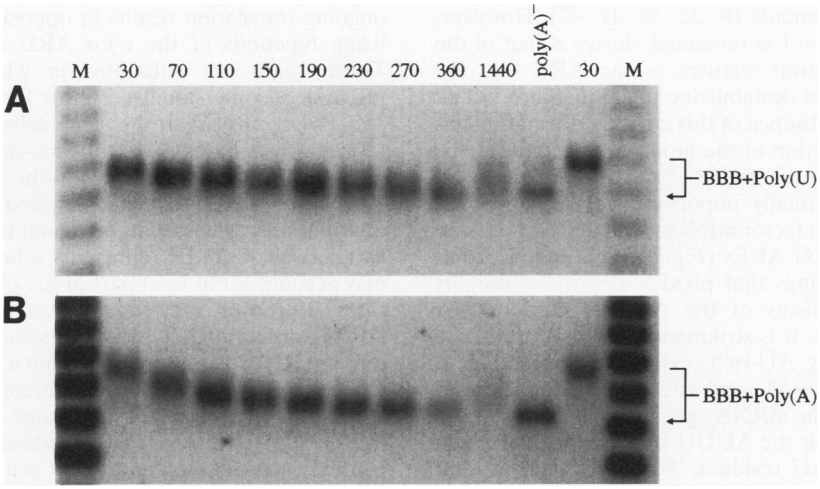

FIG. 7. Decay and deadenylation of β-globin mRNA carrying a poly(U) (A) or poly(A) (B) insertion. Transient transfection, RNA isolation, and time course experiments were carried out as described in the legend to Fig. 6. The times at the top are times (in minutes) after serum stimulation. Poly(A)$^-$ RNA was prepared in vitro by treating the RNA sample from the 30-min time point with RNase H in the presence of oligo(dT) for poly(U) insertion. For poly(A) insertion, a deoxyoligonucleotide-containing sequence complementary to the last 19-nt sequence immediately upstream from the polyadenylation site of the rabbit β-globin mRNA was used in the RNase H digestion to generate the poly(A)$^-$ RNA. Therefore, the 19-nt sequence was taken into account when the size of poly(A)$^-$ RNA was calculated. M, 123-bp DNA ladder from BRL used as a molecular size standard.

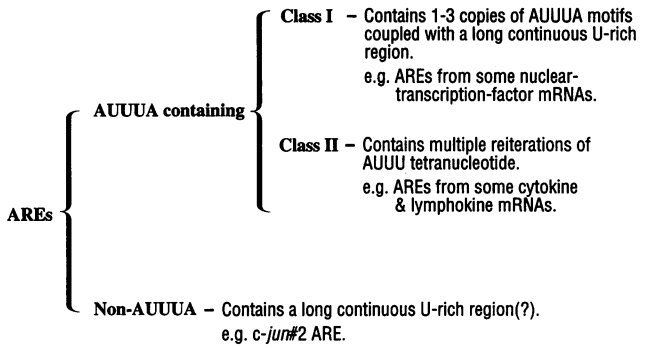

FIG. 8. Classification of the ERG AREs. See the text for details.

insertion by RNase H digestion, the poly(A)$^-$ RNA control lane was generated in a different way. A specific oligomer that hybridizes to the 19-nt region immediately upstream of the poly(A) addition site of the β-globin mRNA rather than oligo(dT) was used for RNase H treatment. Therefore, in this case, the poly(A)$^-$ RNA sample shown in Fig. 7B migrated a little faster than the 1,440-min sample. When the size of poly(A)$^-$ RNA was calculated, the size of the specific oligomer was included. Together, these observations indicated that some specific sequence features other than the adenylate or uridylate richness are necessary for an ARS to function as a potent AU-rich destabilizing element like the c-*fos* ARE.

DISCUSSION

The ARSs were originally noticed in the 3′ UTRs of many transiently expressed lymphokine and cytokine messages (10), which represent one distinct group of ERG mRNAs. Initial studies of one lymphokine mRNA, the granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA, showed that the GM-CSF ARS can function as an RNA-destabilizing element upon its insertion into a stable message (37). More recent experiments have showed that ARSs from the 3′ UTRs of c-*fos*, c-*myc*, and the beta interferon gene can also function as RNA-destabilizing elements (8, 22, 39, 41, 42). However, one fundamental issue that has remained elusive is that of the key sequence and structural features of the ARE that are required to specify its final destabilizing function. Since not all AREs are the same, the absence of this critical information has prevented further elucidation of the mode of action of AREs.

In this report, we have chosen to focus our attention on characterizing the functionally important features of ARSs from nuclear transcription factor mRNAs, which represent one other distinct class of ERG AREs (Fig. 8). This endeavor has led to several novel findings that provide important insights into the overall mechanisms of the ARE-directed mRNA decay in mammalian cells. It is striking to find that four of the eight AUUUA-containing AU-rich regions we tested (Fig. 1, *krox*20#1, *krox*20#2, c-*jun*#1, and *zif*268#1) cannot confer instability on the β-globin mRNA, given the fact that these four ARSs contain not only the AUUUA motifs but also a high percentage (≥70%) of AU residues. Thus, we conclude that the presence of AUUUA motifs even in an AU-rich region does not always guarantee an RNA-destabilizing function for this region. However, it should be noted that the four nonfunctioning ARSs might have RNA-destabilizing abilities when present in their original transcripts with the native context. It is possible that an ARS requires its neighboring sequences in order to function or exert a full destabilizing function (21, 28). For example, the *krox*20#1 and *krox*20#2 ARSs are 34 nt apart in the 3′ UTR of the native *krox*20 mRNA. Although either one alone did not confer instability on the stable β-globin mRNA in this study, when working together in the original *krox*20 context, they may form a functional AU-rich destabilizing element. The other possibility is that there exists some sort of long-range interaction between an ARS and another portion of the mRNA, for instance, the 5′ UTR (31), which is necessary for an ARS to function as an ARE.

A rough classification of AREs and a summary of some of the critical features are shown in Fig. 8. AREs can be divided into two groups: AUUUA containing and non-AUUUA containing. The AUUUA-containing AREs may be further divided into two distinct classes: class I AREs, from some nuclear transcription factor mRNAs, and class II AREs, from some lymphokine and cytokine messages. Our studies show that while the presence of AUUUA motifs is not always sufficient to ensure a destabilizing function for an ARS, for those potent AUUUA-containing AREs the AUUUA motifs are necessary for the destabilizing function. Therefore, the AUUUA motif is a critical but not the only factor in determining the destabilizing function of an AUUUA-containing ARE. AUUUA-containing ARSs that do not possess an RNA-destabilizing ability may lack some important *cis*-acting features which are required to coordinate with the AUUUA motifs for a destabilizing function, e.g., a long stretch of U residues or something like the c-*fos* ARE domain II. Our studies suggest that the presence of one to three copies of the AUUUA motif coupled with a nearby continuous U-rich sequence is a major factor in determining the destabilizing function of the potent AUUUA-containing ARE.

The rationale for dividing the AUUUA-containing AREs into two classes is based on the following observations. First, the AREs from nuclear transcription factor mRNAs, such as c-*fos* and c-*myc* AREs, usually have a few copies of the AUUUA motif coupled with a nearby continuous U-rich sequence, whereas the lymphokine and cytokine mRNAs, such as GM-CSF and interleukin 3 mRNAs, tend to have multiple reiterations of the AUUU tetranucleotide, which in turn account for the high uridylate content (37). Second, three recent reports (1, 23, 35) show that interference with the ongoing translation results in opposite effects on the destabilizing functions of the c-*fos* ARE and the GM-CSF ARE. Third, there are situations in which certain lymphokine mRNAs become stabilized upon T-cell activation, while c-*fos* and c-*myc* mRNAs in the same cells are selectively degraded (26, 36, 37). Finally, a sequence-specific cytoplasmic factor (AU-B) that binds specifically to the GM-CSF ARE but not to the c-*myc* ARE has been identified in T cells (6, 7). These observations suggest that the structural distinction between the two classes of AREs represents a bona fide difference which may account for at least part of the observed differential decay rates. Moreover, they suggest that the two distinct classes of AREs target mRNA decay through different pathways or direct mRNA degradation through a final convergent pathway whose activity depends on differential interactions between specific *cis*- and *trans*-acting factors.

One surprising result is the finding that the c-*jun*#2 ARE contains no AUUUA motifs but can still function as a potent RNA-destabilizing element. Interestingly, the c-*jun*#2 ARE-directed decay also followed a biphasic pattern consistent with the two-step mechanism. This observation has at least two important meanings. First, it shows that the presence of the AUUUA motif is not an absolute requirement for an AU-rich region to function as an RNA-destabilizing element. Second, it suggests that there exists sequence redundancy or a feature(s)

in the c-*jun*#2 ARE that is functionally equivalent to the AUUUA motifs. The question then becomes whether the c-*jun*#2 ARE-mediated decay involves a *trans*-acting factor(s) distinct from those involved in the c-*fos* ARE-directed decay and how the various interactions between these different *trans*- and *cis*-acting factors direct mRNA decay through the same two-step pathway. One noticeable sequence feature in the c-*jun*#2 ARE is the presence of several copies of the GUUUG motif (Fig. 1). It will be interesting to see whether the GUUUG motif is functionally equivalent to the AUUUA motif. In addition, it should also be noted that this non-AUUUA ARE also contains a couple of long continuous U-rich regions, which is a critical characteristic shared by the AUUUA-containing AREs. Elucidation of these critical sequence features of the c-*jun*#2 ARE as well as those of other non-AUUUA AREs is likely to yield further insight into the general mechanism of ARE-directed mRNA decay.

In this study, we report for the first time the identification of an RNA decay-enhancing element (RDE element) which has a decay-enhancing effect on various ERG ARSs. Previously, we showed that the c-*fos* ARE consists of two functionally and structurally distinct domains, termed domain I and domain II (12) (Fig. 1). The presence or absence of domain II can cause a twofold difference in the destabilizing ability of domain I (Table 1) (12). In the same study, we further showed that various decay-impeding effects caused by mutations introduced into domain I can be readily alleviated by fusing domain II immediately downstream of the corresponding mutant domain I. When domain II was fused immediately upstream of domain I, i.e., when the relative positions of domain I and domain II were reversed, domain II retained its decay-enhancing effect. Remarkably, by fusing the c-*fos* ARE domain II immediately downstream of 10 different, heterologous AU-rich regions, we demonstrated that domain II has a two- to sixfold-enhancing effect on the destabilizing function of these heterologous AREs, regardless of whether they carry AUUUA motifs. Taken together, these results lead to the identification of an RDE element. The other important feature of this enhancer-like element is that it can enhance not just the first step in the proposed two-step decay mechanism (Table 1): in most cases, both steps were enhanced. This finding is consistent with findings from our previous study, in which we showed that the c-*fos* ARE domain II is able to enhance the decay of mRNAs carrying various mutant domains I by accelerating the rates for both steps (12). Its presence increases the deadenylation rate, which in turn accelerates the rate for the first step. Interestingly, its presence apparently increases the extent of removal of the poly(A) tail as well. In most cases, the length of the poly(A) tail carried by the last detectable mRNA in the presence of the RDE element is significantly shorter than that of the same mRNA without this RDE element (Table 1).

The findings that both poly(U) and poly(A) can facilitate somewhat rapid and complete removal of the poly(A) tail have a couple of important mechanistic implications. First, the presence of readily detectable RNA whose poly(A) tail was completely removed suggests that in vivo the extreme 3′ ends of deadenylated mRNAs (RNA bodies) are not readily accessible to nonspecific 3′ exonuclease attack and that recognition of additional distinct *cis*-acting elements, such as AUUUA motifs, may be required for subsequent decay of the mRNA body. Therefore, a corollary of the argument would be that the major role of the poly(A) tail is not just to serve as a passive barrier to protect RNA from cytoplasmic nonspecific exonuclease attack. Rather, its shortening or the process of its shortening per se may serve as a signal for triggering the decay of the mRNA body. Second, the ability of the internal poly(A) sequence to facilitate complete removal of the 3′ poly(A) tail from mRNA supports the model of Lowell et al. (27), which proposes that a poly(A)-binding protein (PAB) hopping between the terminal oligo(A) and an internal PAB binding site within the 3′ UTR may be the mechanism for terminal deadenylation of oligo(A) RNA by a yeast poly(A) nuclease (PAN). With regard to internal poly(U), one can speculate that internal poly(U) complexed with its U-rich-sequence-binding proteins (6, 7, 43) may be necessary for PAB hopping or may help to recruit PAN-like nuclease to the RNA for terminal deadenylation.

One could speculate that different AREs may carry different essential but functionally redundant sequence features, such as AUUUA motifs and AU richness, necessary for the assembly of a decay apparatus. The interactions between the c-*fos* ARE domain II or other U-rich sequences and the cognate RNA-binding proteins (6, 7, 43) may enable basic machineries and decay factors to make initial contacts with the AREs that start the assembly of the decay apparatus. Given the observations that poly(A) tails can stimulate translation initiation at the 5′ end of a message (16, 30, 34) and that deadenylation in yeast cells leads to decapping-dependent 5′ exonuclease digestion of the mRNA body (13, 29), it is conceivable that formation of a messenger ribonucleoprotein (mRNP) structure involving the 5′ end, 3′ end, and ARE of an mRNA may be necessary to coordinate the communications among them that will lead to the observed two-step mechanism for ARE-directed mRNA decay. One challenge will be to identify and characterize such an mRNP complex and address the question of how the degradation activities may occur within the mRNP structure. Our investigations of the mechanisms and key structural features necessary for specifying the final destabilizing function of the ARE provide an important experimental basis for further delineation of these critical issues.

ACKNOWLEDGMENTS

We thank Jack DeMoss for critical reading of the manuscript and valuable comments, Gary Brewer and Philip Leder for providing plasmids, Tsuey-Ming Chen for technical assistance, and Beto Zúñiga for expert photography and artwork.

This work was supported by NIH grant GM-46454 (to A.-B.S.). A.-B.S. is the recipient of an American Cancer Society Junior Faculty Research Award.

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